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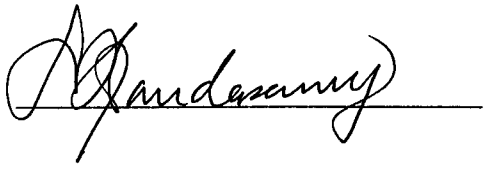

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13. ABSTRACT *(Maximum 200 Words)*
Investigating methylation events associated with estrogen receptor gene regulation will enhance our understanding of hormone resistance in breast cancer. Thus, studies were undertaken to examine the role of DNA methyl-transferase (DMT) in the progression of breast cancer to a hormone-insensitive phenotype. We have found that DMT protein levels tightly correlate with S phase fraction in ER-positive cells, whereas ER-negative cells express DMT throughout the cell cycle. Stable transfection of MCF7 cells with FGFs led to increased DMT expression that could not be accounted for by a shift in S phase fraction. However, acquisition of an estrogen-independent phenotype, even in conjunction with elevated DMT levels, was not sufficient to promote ER gene silencing *via* methylation. These results indicate that multiple steps are required for *de novo* methylation of the ER CpG island. However, we found that reducing expression of DMT protein can lead to a transient changes in ER CpG island methylation, suggesting that overexpression of DMT may be necessary for maintenance of aberrant methylation patterns. In examining primary breast tumor tissue, we found that ER CpG island methylation can take place at a very early stage of breast cancer progression (DCIS), but the incidence increases with disease progression to a metastatic phenotype

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Sharyl Nass 9-28-99

PI - Signature **Date**

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Introduction

A major challenge in improving the treatment of breast cancer is understanding and overcoming resistance to endocrine therapy. At the time of diagnosis, about one third of human breast cancers lack estrogen receptor (ER), a phenotype associated with poor prognosis. ER negative tumors are much less amenable to hormonal therapy with agents like the antiestrogen tamoxifen and are associated with shorter disease-free survival. However, the molecular mechanisms underlying this lack of estrogen receptor expression are poorly understood. Recent findings suggest that epigenetic changes may be important for tumor initiation or progression. Abnormal methylation of CpG islands has been associated with inhibition of expression for a variety of tumor suppressor genes, and a similar phenomenon may block ER gene expression in breast cancer. A CpG island in the promoter region of the ER gene is extensively methylated in ER-negative breast cancer cells, but is umethylated in normal breast cells. Furthermore, expression of the enzyme which catalyzes cytosine methylation, DNA methyltransferase (DNA MTase) is significantly elevated in ER-negative breast cancer cell lines compared to ER-positive lines. Thus, DNA methylation may play a role blocking ER expression as some breast cancers progress to an aggressive, hormone insensitive phenotype.

The purpose of these studies is to test the hypothesis that an increase in DNA MTase expression may promote the progression of breast cancer to a hormone independent phenotype by inhibiting expression of genes, such as the estrogen receptor gene, which would otherwise suppress that phenotype. Relatively little is understood about the contribution of epigenetic changes, such as methylation, to the progression of cancer. Therefore, defining the role of gene methylation in breast cancer could greatly enhance our understanding of the basic changes involved in the disease and may suggest novel therapeutic approaches. Specifically investigating methylation events associated with estrogen receptor gene regulation will enhance our understanding of hormone resistance in breast cancer. Identifying other genes that are silenced in association with an estrogen-independent phenotype may greatly improve our understanding of breast cancer progression.

Body of Report

The work on this project is currently on schedule. All components of the Statement of Work with projected finish dates during Months 1-12 have been accomplished, as flollows. A fragment of human DNA MTase was cloned into the Bluescript plasmid such that riboprobes can be synthesized in both the sense and antisense orientation. This allows for detection of both the endogenous mRNA and the antisense RNA expressed by the expression plasmid. The tetracycline repressible retroviral vector pBPSTR-1 was found to be ineffectual for inducing significant changes in gene expression in breast cancer cells. Therefore, the full length human DNA MTase cDNA was cloned into the heavy metal-inducible plasmid pSARMTneo (provided by P. Morin) as was described in the methods section of the proposal. Bacterial colonies were isolated with the cDNA in both the sense and antisense orientation.

MDA-MB-231 cells were initially transfected with a plasmid containing a truncated form of the human DNA Mtase cDNA in the antisense orientation under control of the CMV constitutive promoter (provided by P. Vertino). Several clones were identified with significantly reduced DMT expression. After several passages in culture, two cell lines exhibited a partially reversion of the methylated phenotype in the ER CpG island. This phenotype was not stable however, as the cell lines eventually reverted to a completely methylated ER CpG island. We believe that such transience in the phenotype is most likely due to selection against cells that re-express the estrogen receptor. Transfection of 231 cells with the full length antisense expression vector described above (pSARTMTneo) was not effective in reducing DMT protein expression.

As an alternative to the antisense transfection approach, a DMT antisense oligonucleotide has been synthesized. Preliminary studies indicate that a 48 hour exposure to the antisense oligo (50 nM) in the presence of Lipofectin (Gibco) is effective in dramatically reducing DMT protein levels in MDA-231 cells. Long term effect of the oligos on growth and survival of the cells, as well as the methylation status of the ER CpG island are currently being investigated.

The full length inducible sense vector (pSARTMTneo-hDMT) has been stably transfected into a subline of the MCF7 breast cancer cell line. Fifteen colonies were selected for resistance to G418 and are currently being screened for elevated DMT protein expression (by Western blot) following a 48 hour exposure to $ZnSo_4$.

Additional studies, beyond those stated in the SOW, were undertaken in order to further elucidate the role of DMT overexpression in breast cancer progression. It is not clear whether CpG island methylation initiates or maintains ER gene silencing, or whether aberrant methylation intrinsically accompanies the transition to an estrogen independent, ER-negative phenotype. If deregulation of DMT is necessary to initiate or maintain aberrant methylation of the ER CpG island, then examining the regulation of DMT expression in breast cancer may greatly improve our understanding of breast cancer progression. We therefore studied the expression of DMT in three panels of established human breast cancer cell lines that potentially represent different stages of breast cancer progression (estrogen-dependent ER-positive cell lines, estrogen-independent ER-positive lines, and ER-negative lines. (See Nass et al., in Press, Oncogene, 1999 in the appendix).

DMT expression was tightly correlated with S phase fraction in ER-positive cells, while ER-negative cells expressed DMT throughout the cell cycle. Thus, breast cancer cells may acquire characteristics that allow them to escape normal cell cycle-dependent regulatory controls on DMT expression during the process of tumor progression. For these studies, a new method was developed to facilitate co-incident detection of cell cycle phase and relative DMT protein expression. Fixed nuclei were analyzed by flow cytometry following immunohistochemical staining of DMT protein with a rabbit polyclonal antibody and a FITC conjugated secondary antibody, and chemical staining of the DNA with propidium iodide.

We also found that the level of $p21^{CIP1}$, which disrupts DMT binding to PCNA, was inversely correlated with DMT levels in breast cancer cells. In addition, activation of peptide growth factor signaling pathways, which is common in breast cancers, led to increased DMT expression in ER-positive MCF7 cells without a concomitant change in S phase fraction. Furthermore, acquisition of an estrogen independent phenotype in MCF7 cells, even in conjunction with elevated DMT expression, was not inherently accompanied by aberrant methylation of the ER gene. These data imply that multiple steps are required for *de novo* methylation of the ER CpG island.

It is also currently unclear when, during malignant progression of ductal breast carcinoma, aberrant methylation of the ER CpG islands begins and whether the incidence of such methylation increases with advancing disease. Therefore, we evaluated a total of 111 ductal

breast carcinomas for the incidence of ER CpG island methylation in *in situ*, (DCIS), invasive, and metastatic lesions. (See Nass et al., manuscript in preparation, 1999 in the appendix). From these studies, we concluded that methylation of the ER CpG island can occur early during progression. About 30% of DCIS samples examined show evidence of such methylation . In addition, the incidence of aberrant ER CpG island methylation increases with progression from DCIS to invasive carcinoma to metastatic disease. Furthermore, abberrant methylation of the ER gene was a specific event – it does not necessarily take place concurrently with methylation of other CpG islands. However, coincident methylation of the ER and E-cadherin genes increased with progression from DCIS to metastatic breast cancer.

Appendix 1

Key Research accomplishments

The following tasks have been completed:

- Riboprobe construction:
  A fragment of human DNA MTase was cloned into the Bluescript plasmid such that riboprobes can be synthesized in both the sense and antisense orientation.
- Construction of Vectors:
  The full length human DNA MTase cDNA was cloned into the heavy metal-inducible expression plasmid pSARMTneo (provided by P. Morin) in both the sense and antisense orientation.
- Transfections:
  MDA-MB-231 cells were initially transfected with a plasmid containing a truncated form of the human DNA Mtase cDNA in the antisense orientation under control of the CMV constitutive promoter (provided by P. Vertino). Several clones were identified with significantly reduced DMT expression. After several passages in culture, two cell lines exhibited a partially reversion of the methylated phenotype in the ER CpG island. Transfection of MDA-231 cells with the full length antisense expression vector described above (pSARTMTneo) was not effective in reducing DMT protein expression.

  The full length inducible sense vector (pSARTMTneo-hDMT) has been stably transfected into a subline of the MCF7 breast cancer cell line. Fifteen colonies were selected for resistance to G418 and are currently being screened for elevated DMT protein expression following exposure to $ZnSo_4$.
- Oligos:
  As an alternative to the antisense transfection approach, a DMT antisense oligonucleotide has been synthesized. Preliminary studies indicate that exposure to the antisense oligo is effective in dramatically reducing DMT protein levels in MDA-231 cells.

- DMT-Cell cycle analysis:
  A new method was developed to facilitate co-incident detection of cell cycle phase and relative DMT protein expression. DMT expression was tightly correlated with S phase fraction in ER-positive cells, while ER-negative cells expressed DMT throughout the cell cycle.
- Analysis of DMT and p21 expression
  The level of $p21^{CIP1}$, which disrupts DMT binding to PCNA, was inversely correlated with DMT levels in breast cancer cells.
- Effect of growth factor overexpression on DMT expression
  Activation of peptide growth factor signaling pathways led to increased DMT expression in ER-positive MCF7 cells without a concomitant change in S phase fraction.
- Analysis of DMT expression and ER methylation during progression from ER+ to ER- status
  Acquisition of an estrogen independent phenotype in MCF7 cells, even in conjunction with elevated DMT expression, was not inherently accompanied by aberrant methylation

of the ER gene. These data imply that multiple steps are required for *de novo* methylation of the ER CpG island.

- Analysis of ER methylation during breast cancer progression
  Methylation of the ER CpG island can occur very early during progression. About 30% of DCIS samples examined show evidence of ER methylation
  The incidence of aberrant ER CpG island methylation increases with progression from DCIS to invasive carcinoma to metastatic disease.
- Analysis of co-incident methylation of ER and Ecadherin in primary breast tumors
  Abberrant methylation of the ER gene is a specific event – it does not necessarily take place concurrently with methylation of other CpG islands.
  Coincident methylation of the ER and E-cadherin genes increases with progression from DCIS to metastatic breast cancer.

Appendix 2

Reportable Outcomes

1. An abstract was presented at the 90th Annual Meeting of American Association for Cancer Research (AACR) in Philadelphia, PA, Abstract #3396. (1999)

2. Two manuscripts were accepted for publication. The first is a review article published *in Hematology/Oncology Clinics of North America* in April of 1999. The second is a primary data paper which was recently accepted for publication in *Oncogene*.

   Nass SJ, and NE Davidson. The Biology of Breast Cancer. Hematology/Oncology Clinics of North America, 13(2):311-332, 1999.

   Nass SJ, AT Ferguson, D El-Ashry, W Nelson, and NE Davidson. Expression of DNA (cytosine-5) methyl-transferase (DMT) and the cell cycle in human breast cancer cells. In press, Oncogene, 1999.

3. One manuscript (primary data) is currently in preparation for submission in the fall of 1999.

   Nass SJ, J.G. Herman, E. Gabrielson, P.W. Iversen, F.F. Parl, S.B. Baylin, N.E. Davidson, and J.R. Graff Aberrant methylation of the estrogen receptor and E-cadherin 5' CpG islandsincreases with malignant progression in human breast cancer (in preparation).

Appendix 3A

IN PRESS, *ONCOGENE*, 1999

Expression of DNA methyl-transferase (DMT) and the cell cycle in human breast cancer cells.

Running Title: DMT expression in breast cancer

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Key Words: breast cancer, estrogen receptor, DNA methylation, S phase, p21

Abstract

ER-negative breast cancer cells display extensive methylation of the ER gene CpG island and elevated DNA methyltransferase (DMT) expression compared to ER-positive cells. The present study demonstrates that DMT protein levels tightly correlate with S phase fraction in ER-positive cells, whereas ER-negative cells express DMT throughout the cell cycle. In addition, levels of $p21^{CIP1}$, which disrupts DMT binding to PCNA, are inversely correlated with DMT levels. Therefore increased DMT expression in ER-negative cells is not simply due to elevated S-phase fraction, but rather to more complex changes that allow cells to escape normal cell cycle-dependent controls on DMT expression. Because ER-negative breast tumors often have activated growth factor pathways, the impact of these pathways on DMT expression was examined in ER-positive cells. Stable transfection with FGFs led to increased DMT expression that could not be accounted for by a shift in S phase fraction. Elevated DMT protein expression in FGF-transfectants was accompanied by a significant decrease in p21, again suggesting a reciprocal relationship between these two proteins. However, acquisition of an estrogen-independent phenotype, even in conjunction with elevated DMT levels, was not sufficient to promote ER gene silencing *via* methylation. These results indicate that multiple steps are required for *de novo* methylation of the ER CpG island.

Introduction

One major challenge in improving the treatment of breast cancer is understanding and overcoming resistance to endocrine therapy. Approximately one third of human breast cancers lack estrogen receptor a (ER). These ER-negative tumors rarely respond to hormonal therapy with agents like the antiestrogen tamoxifen and are associated with shorter disease-free survival (McGuire, 1978; Samaan *et al*, 1981; Early Breast Cancer Trialists Collaborative Group, 1998). Therefore, defining how and why tumors become ER-negative is a critical step for improving breast cancer therapeutic outcome. The molecular mechanisms underlying lack of estrogen receptor expression are poorly understood, but it has been hypothesized that ER-negative breast cancer cells may be derived from ER-positive cells that have acquired the ability to grow independently of estrogen and have lost expression of the gene.

Recent findings suggest that abnormal methylation of the ER gene CpG island may be important for silencing ER gene expression as some breast cancers progress to an aggressive, hormone insensitive phenotype (Ottaviano, *et al*, 1994;, Lapidus *et al*, 1996, 1998; Ferguson *et al*, 1995). CpG islands are cytosine-guanosine rich areas located in the 5' regulatory region of some genes (Bird, 1986). Methylation of a CpG island results in transcriptional silencing of the associated gene, either through direct effects or *via* a change in chromatin conformation that inhibits transcription (Kass *et al*, 1997). In normal somatic cells, CpG islands are usually unmethylated except for genes on the inactive X chromosome and some imprinted genes (Li, *et al*, 1993). In contrast, cancer cells often display anomalous patterns of DNA methylation, with site specific hypermethylation in CpG islands and hypomethylation of bulk genomic DNA (reviewed in Laird and Jaenisch, 1996; Counts and Goodman, 1995).

The CpG island in the promoter region of the ER gene is extensively methylated in established ER-negative breast cancer cell lines and primary tumors, but remains unmethylated in normal tissues and ER-positive breast cancer cell lines. Furthermore, treatment of the ER-negative cell line MDA-MB-231 with an inhibitor of DNA methylation results in demethylation of the ER CpG island and restores expression of a functional estrogen receptor (Ferguson *et al*, 1995). However, it is not clear whether CpG island methylation initiates or maintains ER gene silencing, or whether aberrant methylation intrinsically accompanies the transition to an estrogen independent, ER-negative phenotype.

It is possible that abnormal methylation patterns result from overexpression of the enzyme that catalyzes cytosine methylation, DNA (cytosine-5) methyl-transferase (DMT). Expression of DMT is required for normal maintenance methylation, but changes in enzyme expression or activity may also promote *de novo* changes in a cell's methylation patterns. Increased expression of DMT is an early event in two experimental models of cancer (Belinsky *et al*, 1996; Miyoshi *et al*, 1993), and overexpression of DMT can promote a transformed phenotype in NIH 3T3 cells (Wu *et al*, 1993). In ER-negative breast cancer cell lines, DMT RNA and protein levels are significantly elevated compared to ER-positive cell lines (Ottaviano *et al*, 1994; Ferguson *et al*, 1997), but little is known about the regulation of DMT expression and activity in breast cancer. If deregulation of DMT is necessary to initiate or maintain aberrant methylation of the ER CpG island, then examining the regulation of DMT expression in breast cancer may greatly improve our understanding of breast cancer progression.

We therefore studied the expression of DMT in three panels of established human breast cancer cell lines that potentially represent different stages of breast cancer progression (estrogen-dependent ER-positive cell lines, estrogen-independent ER-positive lines, and ER-negative lines).

We found that DMT expression was tightly correlated with S phase fraction in ER-positive cells, while ER-negative cells expressed DMT throughout the cell cycle. Thus, breast cancer cells may acquire characteristics that allow them to escape normal cell cycle-dependent regulatory controls on DMT expression during the process of tumor progression. Activation of peptide growth factor signaling pathways, which is common in breast cancers, led to increased DMT expression in ER-positive MCF7 cells without a concomitant change in S phase fraction. However, acquisition of an estrogen independent phenotype in MCF7 cells, even in conjunction with elevated DMT expression, was not inherently accompanied by aberrant methylation of the ER gene. Together, these data imply that multiple steps are required for *de novo* methylation of the ER CpG island.

Materials and Methods

Cell Lines

Four ER-positive (MCF-7/WT, ZR-75-1, T47D, and MDA-MB-134) and 6 ER negative (MCF-7/Adr, Hs578t, MDA-MB-231, MDA-MB-435, MDA-MB-453, and MDA-MB-468) human breast cancer cell lines were acquired and routinely maintained as previously described (Ottaviano *et al*, 1994). Eight additional cell lines derived from the ER-positive MCF-7 line were also used in these studies. Raf14c (overexpressing a constitutively active form of Raf) and its vector control, HCopoolc, as well as MKL4 and α18 cells (which overexpress FGF4 and FGF1 respectively) and their vector control, MCN4 were provided by Dr. Dorraya El-Ashry and Dr. Francis Kern of the Lombardi Cancer Center and Southern Research Institute, respectively (El-Ashry *et al*, 1997; Kern *et al*, 1994). These transfected lines have acquired estrogen independent growth as a result of their specific gene overexpression and are grown continuously in the absence of estrogen. The MCN4 and HCopoolc cell lines were selected *in vitro* for estrogen independent growth over a period of about nine months (El-Ashry *et al*, 1997; Kern *et al*, 1994). MCF-7/MIII, MCF-7/LCCI, and MCF-7/LCC2 (Clarke *et al*, 1994) were a gift from Dr. Robert Clarke (Lombardi Cancer Center, Washington DC) and were included in the study as a model of progression to hormone independence. The MIII and LCC1 lines were sequentially selected for estrogen-independent growth in nude mice, and LCC2 was derived from LCC1 by selection for resistance to tamoxifen *in vitro*. All estrogen independent MCF7 derivatives were grown in IMEM without phenol red supplemented with 10% serum that had been charcoal-stripped to remove all steroid hormones (CCS, HyClone Laboratories, Logan, Utah).

FACS Analysis

Cells were plated in 100-mm tissue culture dishes concurrently for cell cycle analysis by FACS and for Western analysis. Two days later, exponentially growing cells were harvested at about 70% confluence. Nuclei were isolated and stained with propidium iodide (PI) for cell cycle analysis according to the method of Vindelov *et al* (1983).

To determine the cell cycle distribution of cells expressing DMT, nuclei were fixed and stained by immunofluorescence for DMT along with PI. Growing cells were harvested by trypsinization and then swelled in HSSE buffer (20 mM HEPES, pH 7.0, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride) on ice for 20 minutes. Cells were lysed in a dounce homogenizer and nuclei were pelleted by centrifugation. The nuclei were resuspended in 0.5 M sucrose/HSSE, layered on a cushion of 1.5 M sucrose/HSSE, and centrifuged for 20 min at 13,000g. Isolated nuclei were then fixed with 3% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes on ice, washed with PBS, and stored at -70 prior to FACS analysis.

The nuclei were resuspended in permeabilization buffer (PBS with 4% goat serum and 0.1% Triton X-100), washed with PBS, and incubated with 1 μg/ml primary DMT antibody (see below) in PBS with 1% BSA for 2 hr at room temperature (RT). Following washes in PBS/1% BSA, the nuclei were incubated for 1 hr at RT with an FITC-conjugated anti-rabbit secondary antibody (Sigma) diluted 1/500 in PBS/1% BSA. Nuclei were washed again in PBS and then stained with PI as above. FACS analysis was repeated at least three times for all cell lines examined.

Preparation and characterization of anti-DMT antiserum

With the help of Research Genetics, Inc., rabbit polyclonal antiserum was raised against a peptide derived from the N-terminal region of DMT (NH_3-MADANSPPKPLSKPRTPRRS-COOH). The peptide was conjugated to KLH and used for rabbit immunization and boosting. The resultant antiserum against the peptide recognized a single polypeptide at the expected molecular weight on an immunoblot of proteins derived from a variety of human cell lines, and depleted DNA methyltransferase activity from nuclear extracts (not shown). Affinity purified antibody was used for FACS and western analysis.

Western Analysis

Cells were plated as described above. Two days later, total cell lysates were prepared. Cells were washed with cold PBS and then scraped into cold lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10 μg/ml PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). After a 10 minute incubation on ice, lysates were spun for 10 minutes in a cold microcentrifuge to remove cellular debris and were frozen at -70 °C. Proteins were boiled and separated by SDS-PAGE and then transferred to nitrocellulose membranes. For DMT analysis, 75 μg of protein from each sample were separated on 6.5% polyacrylamide gels. For PCNA and p21, 25 μg of protein were run on 14% gels. Blots were blocked in 7% BSA in Tris-buffered saline with Tween-20 (TBST; 10 mM Tris, pH 7.4, 150 mM NaCl, 1% Tween-20) for 1 h at room temperature and then incubated in TBST with 1% BSA and the following antibodies (1 μg/ml): DMT (polyclonal), PCNA (monoclonal, Oncogene), or p21 (monoclonal, Oncogene). Proteins were visualized with an HRP-linked second antibody (1/5000 in TBST with 1% BSA) and a chemiluminescent detection system (Pierce, Rockford, IL). India ink staining of the membranes demonstrated equal loading and transfer of the samples.

Densitometry and Statistical Analysis

Western band intensities were quantitated with IPLab Gel 1.5 (Scanalytics, Fairfax, VA). Relative protein levels were calculated relative to the values obtained for the parental MCF-7 line that was assigned an arbitrary value of 1. Correlations between relative DMT protein levels and the other variables examined (% cells in S, G1, or G2/M phase, and relative p21 or PCNA protein levels) were calculated with Statview 4.02. Significance was set at $p<0.05$.

Methylation specific PCR

ER CpG island MSP was performed as previously described (Lapidus, *et al*, 1998). Controls lacking DNA were carried out with each set of PCR reactions. 10 μl of total reaction was run on a 3% TBE/agarose gel, stained with ethidium bromide, and visualized by UV light. Eagle Eye technology (Stratagene) and standard graphic software packages were utilized to prepare figures.

Results

DMT levels correlate with S phase fraction in ER-positive, but not ER-negative cells

It had been previously demonstrated with six breast cancer cell lines that DMT mRNA, protein, and enzyme activity (Ottaviano *et al*, 1994; Ferguson *et al*, 1997), were elevated in ER-negative breast cancer cells compared to ER-positive cells. Because DMT protein is known to be expressed primarily during the S-phase of the cell cycle in normal cells (Szyf *et al*, 1985, 1991), we first determined whether the elevated DMT protein level in ER-negative cell lines was simply due to a larger S-phase fraction in those cells. Cell cycle distribution and DMT expression were examined during exponential growth in a larger panel of 10 breast cancer cell lines (4 ER-positive and 6 ER-negative). For each cell line, nuclei for FACS analysis and total protein lysates for Western analysis were prepared concurrently. The results of the FACS analysis are shown in Table 1. ER-positive cell lines generally had a higher G_1 fraction and a lower S-phase fraction than the ER-negative lines. Western analysis confirmed that DMT protein levels were also lower in ER-positive than ER-negative cells (Figure 1). However, statistical analysis of these results showed a significant correlation between S-phase fraction and DMT protein level in ER-positive cells ($R=0.991$, $p=0.009$, Figure 2a), but not ER-negative cells ($R=0.06$, $p=0.91$, Figure 2b), indicating that elevated DMT levels in ER-negative cells could not be explained solely by a change in S phase fraction. There was no significant correlation between DMT level and the fraction of cells in the other phases of the cell cycle (G_1 or G_2/M).

DMT and p21 are inversely correlated in breast cancer cells

A recent study reported that DMT can bind to PCNA (proliferating cell nuclear antigen), and that $p21^{CIP1}$ can disrupt the association between DMT and PCNA, possibly affecting the activity of these proteins (Chuang *et al*, 1997). We therefore examined the levels of PCNA and p21 in the panel of ER-positive and -negative breast cancer cells (Figure 1). As shown in figure 2C, DMT protein levels were inversely correlated with the level of p21 in breast cancer cells ($R=0.68$, $p=0.031$). Most ER negative cells expressed little or no p21, while expression levels were quite high in most ER-positive cell lines. PCNA expression did not vary greatly among the cell lines, and was not significantly correlated with DMT expression (Figure 1).

ER-negative cells express DMT in all phases of the cell cycle

To determine the cell cycle distribution of DMT-expressing cells, nuclei from exponentially growing cells were harvested, fixed, and analyzed concurrently for PI staining and DMT expression. As shown in figure 3 and Table 2, there was a striking difference between ER-positive and ER-negative cells. About 90% of cells in ER-negative lines expressed detectable amounts of DMT protein, whereas only 40% or less of ER-positive cells contained a significant level of DMT. The most remarkable difference was observed in the G_1 phase of the cell cycle. In ER-positive lines, only about 20% of G_1 cells stained positive for DMT, whereas 80% or more of G_1 cells in ER-negative lines were DMT-positive. Statistical analysis of the data (ANOVA) confirmed a significant difference ($p<0.01$) in the number of DMT-positive cells between ER-positive and ER-negative cell lines.

Estrogen independence does not lead to methylation of the ER CpG island in MCF7 cells

Many ER-negative breast tumors have up-regulated growth factor signaling pathways, possibly as a precursor of estrogen independence to compensate for the loss of estrogen signaling (Dickson and Lippman, 1995). In order to determine whether growth factor pathways might play a role in the overexpression of DMT in breast cancer, the ER-positive cell line MCF7 was treated

with transforming growth factor α (TGFα) prior to protein harvest for Western analysis. Treatment of MCF7 cells with TGFα resulted in a two-fold increase in DMT protein levels within 4 hours (Figure 4). There was no concurrent increase in S-phase fraction within that time frame, as expected (not shown). Therefore, another panel of cell lines (Figure 5) was examined to determine whether progression of MCF7 cells to estrogen independence, *via* selection in estrogen-depleted conditions (CCS) or transfection with growth factors or Raf, was accompanied by increased DMT expression and/or ER gene methylation.

Four sets of MSP primers were used to examine the methylation status of these cell lines at several locations in the ER promoter region. The ER CpG island remained completely unmethylated in all of these transfected and selected MCF7 derivatives (Figure 6). This is in marked contrast to another MCF7 derivative, the MCF7(ADR) line, shown in Figure 1. The MCF7(ADR) cells developed an ER-negative phenotype with extensive methylation of the ER CpG island (Lapidus) following selection for adriamycin resistance (Vickers *et al*, 1988).

Selection for estrogen independence does not lead to deregulation of DMT

We next examined DMT protein levels and cell cycle distribution in these lines. Cell lines that had been selected *in vivo* for estrogen independence (MIII, LCC1, and LCC2) manifested DMT protein levels and S-phase fractions similar to those observed for parental MCF7 cells (Table 1, Figure 7). Vector control lines that had been selected for growth in CCS *in vitro* (MCN4c and HCopoolc) exhibited a comparatively low S phase fraction and expressed a relatively small amount of DMT. More importantly, all MCF7 variant lines selected for growth in the absence of estrogen *in vitro* or *in vivo* showed the same correlation between S phase and DMT level that was observed in the original panel of ER-positive cells (Figure 8a, R=0.858, p=0.03). The two regression lines are nearly parallel, with only a small shift to the left for the MCF7 variant lines, perhaps as a result of growth in CCS rather than FCS.

DMT protein level is elevated in transfected MCF7 cell lines

Constitutive expression of FGF1, FGF4, or Raf led to increased DMT protein levels compared to vector-transfected controls (Figure 7, 8b). Transfection of MCF7 cells with FGF1 (α18) or FGF4 (MKL4) resulted in a 4-fold increase in DMT levels that was not associated with a shift in S-phase fraction (Figure 8b). In contrast, transfection with constitutively active Raf promoted a smaller increase in DMT level in conjunction with a shift in S-phase fraction. In figure 8b, the line showing the relationship between Raf14c and its vector control is parallel to the two regression lines in 8a.

p21 expression in MCF7-derived cell lines

As in the original panel of cell lines, a trend toward inverse correlation between DMT and p21 levels was noted among the MCF7 derivatives, but it did not achieve statistical significance (Figure 8c). However, the two FGF-transfected cell lines (α18 and MKL4) with significantly elevated DMT in the absence of increased S-phase fraction, also showed a substantial decrease in p21 protein level (about 6 fold). As before, PCNA expression was not correlated with DMT levels.

Discussion

About one third of human breast cancers are labeled ER-negative based on hormone binding or immunohistochemical assays. Breast tumors that express ER grow more slowly, are more highly differentiated, and are associated with longer disease free survival than tumors that lack ER (McGuire, 1978; Samaan *et al*, 1981). The treatment outcome of breast cancer is also related to ER expression because ER-positive tumors often respond to endocrine therapy with antiestrogens like tamoxifen (McGuire, 1978; Early Breast Cancer Trialists Collaborative Group, 1998) whereas ER-negative breast cancers are nearly always resistant to endocrine therapy. Defining the mechanism by which breast cancer cells develop an ER-negative phenotype is therefore a crucial step for developing novel therapeutic strategies for this group of patients.

Hypermethylation of the ER gene CpG island is the only molecular change that has been consistently identified with the lack of ER gene expression in ER-negative breast tumors to date (Lapidus *et al*, 1998a,b). However, it is not known how the ER gene becomes aberrantly methylated in these tumors. Transfection studies with human fibroblasts have shown that increased levels of DMT expression can lead to *de novo* methylation of many promoter sequences (Vertino *et al*, 1996). Several studies have also suggested that DMT expression is commonly elevated in cancer cells compared to normal cells of the same tissue type (Laird and Jaenisch, 1996), perhaps providing an explanation for the high frequency of abnormal CpG island methylation in cancer cells.

However, a recent study of colon tumors and matched normal colonic mucosa indicated that DMT expression in tumors is only modestly increased above that seen in normal colon tissue, and the increase in DMT expression was correlated with increased histone H4 expression, a measure of S-phase-specific gene expression (Lee *et al*, 1996). Those results were perhaps not surprising in light of the fact that DMT expression and activity are known to vary with the cell cycle (Szyf *et al*, 1985, 1991). *In vivo*, DMT activity of non-proliferating tissues increases following a mitogenic stimulus with a time course coincident with entry into S-phase (Szyf *et al*, 1985). *In vitro*, DMT enzyme activity and mRNA levels in fibroblasts are both maximal during the S-phase portion of the cell cycle (Szyf *et al*, 1991)

Because DMT levels are elevated in ER-negative human breast cancer cell lines compared to ER-positive lines, it was important to determine whether this difference was due simply to altered cell cycle distribution or to a more complex phenomenon. In ER-positive cells, DMT expression was tightly correlated with S-phase fraction. However, elevated DMT levels in ER-negative cells could not be explained solely by a change in cell cycle progression because DMT levels were not correlated with S phase in these cell lines. Thus, breast cancer cells may acquire characteristics that allow them to escape normal cell cycle-dependent regulatory controls on DMT expression during the process of tumor progression.

An increase in DMT expression in the absence of a concurrent increase in the percentage of cells in S-phase could be achieved in one of two ways. First, DMT protein could simply be expressed at abnormally high levels during S phase. Alternatively, cell cycle dependent regulation of DMT protein expression could be lost or altered, resulting in inappropriate expression of the protein in other phases of the cell cycle. Our results indicate that the latter occurs in breast cancer. We demonstrated a striking difference in cell cycle distribution of DMT-expressing cells between ER-positive and ER-negative cell lines. DMT was expressed during all phases of the cell cycle in ER-negative lines, with nearly all of the cells staining positive for DMT. In contrast, only 40% or

less of ER-positive cells expressed detectable amounts of DMT, and most cells in the G_1 phase were negative for DMT.

The normal association of DMT with the S phase of the cell cycle is not surprising, given the recent studies which reported that DNA methylation takes place concurrently with replication, perhaps *via* DMT binding to PCNA at the replication fork (Chuang *et al*, 1997; Araujo *et al*, 1998). The cell cycle inhibitor $p21^{CIP1}$ can disrupt the association between DMT and PCNA, perhaps affecting the activity of the two proteins (Chuang *et al*, 1997). Furthermore, levels of DMT and p21 proteins were inversely related in normal and SV-40 transformed human fibroblasts. We therefore examined the levels of PCNA and p21 in the panel of ER-positive and -negative breast cancer cells and found that DMT protein levels were inversely correlated with the level of p21 in breast cancer cells. In contrast, PCNA expression did not vary greatly among the cell lines, and was not significantly correlated with DMT expression. It has been suggested (Chuang *et al*, 1997) that the transforming effect of DMT overexpression observed by others (Wu *et al*, 1993) may be due in part to the ability of DMT to compete with p21 for PCNA binding, thereby promoting the G1-S phase transition. By binding to PCNA in place of p21, excess DMT could increase the level of active cyclin-dependent kinases, promoting Rb phosphorylation and thus progression through the cell cycle. If that hypothesis is correct, then the relatively higher S-phase fraction of ER-negative cells may be related to the high ratio of DMT to p21 in these cells. In this scenario, the increased DMT level in ER-negative cells could be a cause, rather than an effect, of a higher S-phase fraction in those cells. However, the question still remains as to how DMT expression is deregulated in ER-negative breast cancer cells.

One potential mechanism for upregulation of DMT is altered transcriptional regulation. The mouse DMT gene has been reported to contain AP1 sites that can regulate its expression (Rouleau *et al*, 1995; Macleod *et al*, 1995). More recently, it was demonstrated that transformation of human fibroblasts by fos, a component of AP1, is dependent upon a fos-induced increase in DMT expression and activity (Bakin and Curran, 1999). Because many ER-negative tumors overexpress growth factor receptors whose signaling pathways include AP1 activation (Dickson and Lippman, 1995), we hypothesized that these pathways may play a role in the upregulation of DMT expression. In fact, the ER-negative breast cancer cell line with the highest level of DMT expression (MDA-MB-468) is known to overexpress the epidermal growth factor receptor (Filmus *et al*, 1985). Peptide growth factors stimulate several intracellular signaling pathways, but it is the Ras kinase pathway that leads directly to activation of the transcription factor AP1. Interestingly, the Hs578t cell line, which expressed DMT at a much higher level than would have been predicted by its relatively low S-phase fraction, has a constitutively active mutant form of Ras (Kraus *et al*, 1984).

Activation of the EGFR in MCF7 cells by treatment with TGFα resulted in a two-fold increase in DMT protein levels, suggesting that growth factor receptor activation could indeed have an impact on DMT expression in breast cancer cells. Therefore, another panel of cell lines was examined to determine whether abnormal activation of growth factor signaling pathways could lead to DMT overexpression and/or ER gene methylation in ER-positive cells. These lines were all derived from the ER-positive MCF-7 line. Three of the lines were stably transfected with expression constructs for constitutively active Raf (El-Ashry *et al*, 1997) or members of the fibroblast growth factor family (Kern *et al*, 1994), all of whose signaling pathways can lead to AP1 activation. These transfected cell lines were chosen because all three have inherent estrogen

independence *in vitro*, but they differ in estrogen receptor status, with only the Raf14c line being ER-negative (Kern *et al*, 1994; El-Ashry *et al*, 1997). The other cell lines were selected for estrogen independent growth (Clarke *et al*, 1994; El-Ashry *et al*, 1997) and were included in the study to determine whether up-regulation of DMT accompanies the acquisition of an ER-independent phenotype in this progression model.

Statistical analysis showed that DMT expression was still correlated with S-phase in the MCF7-derived cell lines which had been selected for growth in the absence of estrogen, suggesting that aberrant expression of DMT does not necessarily accompany the progression to estrogen independence. However, constitutive overexpression of FGF1 or 4, which allows the cells to grow without estrogen stimulation, led to a significant up-regulation of DMT expression that could not be accounted for by a shift in S phase fraction. These results demonstrate that activation of growth factor signaling pathways can have an impact on the regulation of DMT expression. Overexpression of a constitutively active Raf kinase also led to increased DMT expression, but in this case, the change in expression was fully explained by a corresponding increase in S phase fraction. It is not clear why the results were different for the FGF and Raf transfectants, since all three proteins activate a Map kinase cascade. However, growth factors like FGF1 and FGF4 activate several intracellular pathways in addition to the Map kinase pathway. Perhaps activation of multiple pathways is necessary to disrupt the regulation of DMT protein expression in these cells. It is important to note that DMT protein levels have been shown to be regulated at the post-transcriptional level (Szyf *et al*, 1991), so even if DMT transcription can be upregulated *via* Raf or AP1 activation, this may not be sufficient to elevate DMT protein levels. Additional post-transcriptional or post-translational regulatory mechanisms may supersede an increase in transcriptional activity.

The elevated DMT protein expression in FGF transfectants was accompanied by a significant decrease in p21, again suggesting a reciprocal relationship between these two proteins. Interestingly, we have also observed that the DMT/p21 ratio is significantly decreased in MDA-231 cells that are stably transfected with a DMT antisense expression vector. Clones with decreased DMT expression also tended to have elevated p21 protein levels (mean relative ratio of DMT to p21 was 1.7) compared to parental MDA-231 cells or vector-transfected controls (mean relative ratio of 42; unpublished results). The fact that we have observed a similar phenomenon in two very different transfected model systems raises the question as to whether association with PCNA might affect the stability of these proteins. For example, because DMT and p21 compete for the same binding site on PCNA, an increase in DMT expression might promote dissociation of p21 from PCNA, perhaps making p21 more susceptible to ubiquitination and proteosome degradation (Maki and Howley, 1997). A decrease in DMT expression would then be expected to have the opposite effect on p21 stability.

In spite of our previous findings showing a connection between DMT expression and loss of ER expression, the elevated DMT expression in these ER-positive, estrogen independent cells was not sufficient to promote ER gene methylation and loss of ER gene expression. All of the cell lines derived from MCF-7 cells *via* transfection or selection in the absence of estrogen grow in an estrogen-independent manner (Clarke *et al*, 1994; El-Ashry *et al*, 1997; Kern *et al*, 1994). However, all but one (Raf14c) continue to express ER mRNA and protein. The Raf14c cell line does not express detectable levels of ER proteins, but it does express a very small amount of ER mRNA. Furthermore, the ER CpG island remained completely unmethylated in all of these cell lines, including Raf14c. Thus, acquisition of an estrogen-independent phenotype, even in

conjunction with dramatically reduced ER expression in the Raf14c line, was not sufficient to promote ER gene silencing *via* CpG island methylation. These results are not due to an inherent inability of the MCF7 cells to methylate the ER gene. We know that MCF7 cells can acquire the capability to methylate the ER gene and silence its transcription because the MCF7ADR line, which was selected for resistance to adriamycin, is ER negative and shows deregulated DMT expression and extensive ER CpG island methylation (Lapidus *et al*, 1998).

These results suggest that ER gene transcription must first be down-regulated by another mechanism, perhaps *via* altered availability of a critical transcription factor, prior to DNA methylation. In this scenario, aberrant CpG island methylation may act to consolidate and strengthen transcriptional silencing of the associated gene. Although it has not been definitively demonstrated that ER-positive cells progress to ER-negative breast tumors, we propose that such a transition would require the tumor cells to pass through at least three stages during the progression from an estrogen dependent phenotype, to a stable, ER-negative phenotype. These stages would include acquisition of estrogen independence, loss of ER transcription, and finally ER CpG island methylation. It is not yet clear whether aberrant DMT expression precedes ER gene methylation, but our results with the Raf-transfected cell line suggest that loss of ER expression alone is not sufficient to trigger ER gene methylation. Although elevated DMT protein expression in the FGF model system was not sufficient to induce ER CpG island methylation, deregulation of DMT expression or activity may still be a prerequisite for *de novo* methylation of the ER gene in breast cancer cells. Alternatively, an increase in DMT expression may be necessary to maintain the abnormal methylation pattern after the gene has been silenced. These critical questions will be addressed in future studies.

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Table 1: FACS analysis of human breast cancer cell lines during exponential growth. Representative results for one experiment are shown.

Cell Line	% S phase	% G1 phase	% G2/M
ER-Positive Lines			
MDA-MB-134	11	81	8
ZR75-1	17	71	12
T47D	20	69	12
MCF7	36	52	13
ER-Negative Lines			
MCF7Adr	34	54	12
MDA-MB-231	30	55	15
MDA-MB-468	25	64	11
MDA-MB-435	41	47	12
Hs578t	23	62	15
MDA-MB-453	27	61	13
MCF7 Derivatives			
MCN4	19	68	13
MKL4 (FGF4)	22	66	12
α18 (FGF1)	19	71	11
HCopoolc	18	70	12
Raf14c	26	50	24
MIII	28	60	12
LCC1	39	51	10
LCC2	33	33	34

Figure Legends

Figure 1: Expression of DMT, PCNA, and p21 protein in established human breast cancer cell lines. Whole cell lysates for Western analysis were prepared from a panel of 4 ER-positive and 6 ER-negative cell lines during exponential growth.

Figure 2: Relationship between relative DMT expression and other variables in human breast cancer cell lines. Representative results for one of three experiments are shown. **A:** A tight correlation was observed between DMT protein levels and S-phase fraction in ER-positive cells. **B:** DMT protein level was not correlated with S phase fraction in ER-negative cells. **C:** Relative p21 and DMT protein levels were inversely correlated ($p<0.05$). □, ER-positive lines; O, ER-negative lines.

Figure 3: Concurrent analysis of cell cycle distribution and DMT expression. Fixed nuclei from exponentially growing cells were immunochemically stained with PI as well as an anti-DMT primary antibody and an FITC- conjugated secondary antibody. ER-positive cell lines (ZR75, T47D) are shown on the left. ER-negative cell lines (MDA-MB-231, MDA-MB-468) are on the right. Control = PI + secondary antibody only.

Figure 4: TGFα increases DMT expression in MCF7 cells. Two days after plating, cells were treated with TGFα (20 ng/ml) for the indicated times before harvesting total cell lysates for DMT Western analysis (panel **A**). Panel **B**: Densitometric analysis of DMT bands obtained in panel A. Values are calculated relative to untreated MCF7 cells, which are assigned an arbitrary value of 1. Error bars, S.E. n=3.

Figure 5: Derivation of the MCF7 sublines. Panel **A**: Cells transfected with FGF1, FGF4, or a constitutively active Raf are inherently estrogen-independent as a result of gene overexpression. Vector controls for these lines (MCN4 and HCopoolc) were selected for estrogen independence *in vitro* over a period of about 9 months. Panel **B**: The MIII and LCC1 lines were sequentially selected for estrogen-independent growth in nude mice, and LCC2 was derived from LCC1 by selection for resistance to tamoxifen *in vitro.*

Figure 6: The ER CpG island remains unmethylated in estrogen independent lines derived from the ER-positive human breast cancer cell line MCF-7, as shown in Figure 5. Methylation specific PCR was used to assess the methylation status of multiple CpGs within the island. Representative results from one primer set are shown, since four different primer sets gave identical results. u=primers specific for unmethylated DNA, m=primers specific for methylated DNA. Water served as a negative control and DNA from MDA-MB-231 cells served as a positive control for the methylated reaction.

Figure 7: Expression of DMT, PCNA, and p21 protein in a panel of estrogen independent cell lines derived from MCF7 cells, as shown in Figure 5. Whole cell lysates for Western analysis were prepared from exponentially growing cells. All cell lines were grown in CCS except untransfected MCF7 cells.

Figure 8: Relationship between relative DMT expression and other variables in MCF-7 variant cell lines. Representative results from one of three experiments are shown.

A: DMT levels were positively correlated with S-phase fraction among untransfected or control vector-transfected cell lines, similar to results obtained for ER positive cell lines in Figure 2 (shown here as a dotted line). **B:** Transfection of MCF7 cells with FGF1 (α18) or FGF4 (MKL4) resulted in a 4-fold increase in DMT levels that was not associated with a shift in S-phase fraction, while overexpression of constitutively active Raf promoted a parallel increase in DMT level (1.6 fold) and S-phase fraction. Lines show the relationship between transfected lines and their specific controls. **C:** A trend toward inverse correlation between relative p21 and DMT expression levels is noted ($p>0.05$).

Appendix 3B

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Aberrant methylation of the estrogen receptor and E-cadherin 5' CpG islands increases with malignant progression in human breast cancer[1]

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Running Title: DNA methylation in breast cancer progression

Key Words: epigenetics, DCIS, invasive ductal carcinoma, metastasis, methylation-specific PCR

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[3]Abbreviations: ER, estrogen receptor-α; E-cad, E-cadherin; MSP, methylation-specific PCR; DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; MDC, metastatic ductal carcinoma; LA, locally advanced

ABSTRACT

Loss of expression for both the estrogen receptor and E-cadherin genes has been linked to disease progression in human ductal breast carcinomas and has been associated with aberrant 5' CpG island methylation. To assess when, during malignant progression, such methylation begins and whether such methylation increases with advancing disease, we have surveyed 111 ductal carcinomas of the breast for aberrant methylation of the ER and E-cad 5' CpG islands. Hypermethylation of either CpG island was evident prior to invasion, in approximately 30% of *in situ* lesions, and increased significantly to nearly 60% in metastatic lesions. Coincident methylation of both CpG islands also increased significantly from approximately 20% in DCIS to nearly 50% in metastatic lesions. Furthermore, in all cases, the pattern of methylation displayed substantial heterogeneity, reflecting the well-established, heterogeneous loss of expression for these genes in ductal carcinomas of the breast.

INTRODUCTION

Human breast carcinomas most frequently evolve from the epithelial lining of the terminal mammary ducts as ductal carcinoma in situ (DCIS) that may progressively become invasive and ultimately metastatic (Beckmann). The transformation of normal mammary epithelial cells into a carcinoma, and the subsequent progression to invasion and metastasis, involves the accumulation of numerous genetic "hits", including the activation or amplification of dominant oncogenes and the deletion or inactivating mutation of key tumor suppressor genes (Heppner). It has recently become evident that tumor suppressor genes may also be transcriptionally silenced in association with aberrant promoter-region, CpG island methylation (Baylin 98; Jones and Laird, 99).

The estrogen receptor α (ER^3) gene and the E-cadherin (E-cad) gene have frequently been implicated in the initiation and/or progression of human breast cancer. Loss of expression of either gene has been associated with poorly differentiated tumors and poorer prognosis (McGuire, Bracke, Charpin, Gupta, Lipponen, Siitonen). Furthermore, several studies have reported an association between E-cad and ER expression in breast tumors (Siitonen, Charpin, Lipponen). In breast cancer cell lines and primary human breast tumors, loss of ER and E-cad expression has been associated with aberrant 5' CpG island methylation (Ottaviano 94, Lapidusx2, Graff 95, 97 ,hopefully, PNAS 99). It is currently unclear when, during malignant progression of ductal breast carcinoma, aberrant methylation of these CpG islands begins and whether the incidence of such methylation tracks with advancing disease for either or both genes. Therefore, we have evaluated a total of 111 ductal breast carcinomas for the incidence of CpG island methylation for these two key suppressor genes in *in situ*, invasive, and metastatic lesions. Our results indicate that the aberrant methylation of either CpG island begins early, prior to invasion, and increases with metastatic progression. Coincident methylation of both CpG islands also increases with progression, suggesting that the malignant progression of ductal breast carcinoma involves the accumulation of multiple epigenetic "hits".

MATERIALS AND METHODS

Tissue Samples. A total of 111 human breast tumor samples identified as ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC), and locally advanced or metastatic ductal carcinoma (LA/MDC) were obtained from the Department of Pathology at Johns Hopkins

University School of Medicine and from the Department of Pathology at Vanderbilt University Hospital. 75% of the LA/MDC samples were derived from lymph nodes, while the remaining 25% consisted of samples from a variety of sites including the chestwall, bone, and lung. Two cases of recurrent breast cancer following lumpectomy were also included. In the case of DCIS, samples were carefully microdissected prior to DNA isolation to avoid sample contamination with other cells.

Cell Lines. Two human breast cancer cell lines were used as controls for methylation assays. MCF-7 cells express both estrogen receptor and E-cadherin, and the CpG islands of both genes are unmethylated in this cell line. The MDA-MB-231 cell line exhibits extensive methylation of the estrogen receptor and E-cadherin gene CpG islands and the cells lack expression of the two genes at both the mRNA and protein level (Ottaviano, Lapidus 98,Graff 95, 97). The cell lines were routinely maintained as previously described (Ottaviano).

DNA Isolation. DNA was isolated from the tissues and cell lines as previously described (Graff 99, Lapidus98). DNA samples were labeled with a coded identification number so that MSP analysis could be performed and analyzed without knowledge of the sample origin.

Methylation-Specific PCR. ER and E-cadherin 5' CpG island MSP was performed on sodium bisulfite-treated DNA as previously described (Lapidus 98, Graff 96). The ER primers (primer set #5, Lapidus 98) target a region of the gene about 400 bp downstream from the transcription start site near a Not1 site (Lapidus 97). MSP primers spanning the transcription start site of E-cad were previously described as Island 3 (Graff '97). Earlier studies showed that methylation in the regions targeted by these primer sets correlated best with loss of gene expression (Graff 97, Lapidus 98). A fraction of the tumor samples in the current study were also analyzed with additional MSP primer sets for the two genes to verify the density of CpG island methylation in these tumors. For many samples, the methylation status of ER and E-cad was assessed concurrently by including primers for both genes in the same reaction (termed duplex PCR).

Statistical Analysis. Any tumor sample that reliably yielded a PCR product in the methylated reaction visible by ethidium bromide staining was considered positive for CpG methylation. The Mantel-Haenszel Chi-Square test for trend was applied to three-by-two tables of tumor type vs. methylation (yes/no) in order to assess the change in percent methylation with increasing tumor progression. Then each pair of tumor types was compared using logistic regression. Significance was set at $p<0.05$.

RESULTS AND DISCUSSION

The estrogen receptor and the homotypic cell:cell adhesion molecule, E-cadherin, both play a role in maintaining the normal differentiated state of the mammary gland epithelium (Bracke; Henderson). Loss of the ER during breast cancer progression is associated with poorer histological differentiation, higher growth fraction, and poorer clinical outcome, and may represent a key mechanism facilitating hormone resistance (McGuire, meta). Similarly, loss of E-cadherin expression has been repeatedly associated with loss of differentiation, increased invasive and metastatic potential, and decreased patient survival (Mareel 95, Bracke, Semb, Lipponen). The transcriptional silencing of both ER and E-cad in human breast cancer has been associated with aberrant promoter-region CpG island hypermethylation. In addition, treatment of human breast cancer cell lines lacking ER and/or E-cad with DNA methyltransferase inhibitor (5-deoxyazacytidine) elicits CpG island demethylation and re-expression of E-cad and ER protein, thereby indicating that aberrant methylation of these CpG islands plays a substantial role

in suppressing transcription of these two key suppressor genes in breast cancer cells (Ferguson, Graff 95).

Because expression of both ER and E-cad is lost in association with aberrant 5' CpG island methylation during breast tumorigenesis, we sought to define the stage of breast tumor progression at which the hypermethylation of these two CpG islands begins, and whether such methylation tracks with advancing disease. We analyzed a total of 111 ductal breast carcinomas comprised of *in situ* lesions (DCIS), invasive, and metastatic cancers by methylation specific PCR (MSP) (Herman 96).

The incidence of CpG island methylation increases with tumor progression.

MSP has previously been used to detect aberrant DNA methylation of several genes, including ER and E-cad, in human cancers (Herman 96, Graff 97, Lapidus, 98). Neither gene is methylated in normal breast epithelia (Graff 95 & 97 , Ottaviano, Lapidus). However, methylation of the two CpG islands was evident in all tumor stages and showed remarkably similar increases during progression from DCIS to metastatic tumors. Methylation of the ER gene was evident in 34% (12/35) of DCIS lesions, while E-cad methylation was evident in 31% (11/35). Coincident methylation was present in only 21% of these DCIS lesions. (Figure 1 and summarized in Table 1). In invasive and metastatic ductal carcinomas (IDC or MDC), the incidence of methylation markedly increased relative to the DCIS lesions. Twenty-five of 48 (52%) IDC samples showed methylation of the ER or E-cad 5' CpG island (Figure 2??, Table 1). Of these 48 samples, 18 (38%) showed distinct, coincident methylation of both CpG islands. Of the locally advanced and metastatic tumor samples, nearly 60% exhibited methylation for each of the CpG islands (Figure 3??, Table 1), while coincident methylation of both CpG islands was apparent in 50% (14/28) of these samples.

These data indicate that the epigenetic inactivation of either gene may occur early, prior to invasion, but increases as cells acquire invasiveness and metastatic potential. The Mantel-Haenzael Chi-Square test for trend demonstrated that the trend toward increased methylation during progression was statistically significant for each gene ($p<0.05$, Table 1). Furthermore, pair-wise comparison of the three tumor types demonstrated that the incidence of methylation in metastatic tumors was significantly higher than in DCIS for both ER (odds ratio=2.96, $p=0.039$) or E-cad (odds ratio=3.37, $p=0.022$). The incidence of methylation in IDC samples was not statistically different from the other two categories, however.

The trend toward increasing coincident methylation of the two genes during progression was also statistically significant ($p=0.019$, Table 1). Thus, the frequency of coincident methylation of both CpG islands increases with advancing disease, suggesting that malignant progression of ductal breast carcinoma involves the accumulation of multiple epigenetic "hits". However, it is important to note that the similarity in the trends for ER and E-cad methylation was not due to complete coincidence of methylation for the two genes. At every stage of progression, the rate of coincident methylation was lower than the incidence of methylation for each individual gene (Table 1). Overall, about 25% of the samples analyzed showed methylation of either ER or E-cad, but not both. These results imply that aberrant methylation of these CpG islands does not simply reflect a generalized increase in CpG island methylation, but may reflect a more specific selection process targeting key suppressor genes.

CpG island methylation is heterogeneous in breast tumors.

In all samples harboring methylation, unmethylated alleles were invariably also evident (Figure 1). For the IDC and LA/MDC samples, which were not microdissected, these unmethylated alleles may reflect the contribution from normal cells in the sample. Alternatively, these alleles may be derived from cancer cells that harbored only unmethylated copies of the E-cad and ER CpG islands. However, this same heterogeneous pattern was evident in the methylated DCIS samples, which were carefully microdissected, suggesting that methylation of these CpG islands in these tumors is heterogeneous. Interestingly, expression studies have routinely revealed that the loss of both E-cad and ER exhibits distinct heterogeneity in ductal breast carcinomas (Siitonen, Bracke, Lipponen, Walker). Thus, the heterogeneous patterns of CpG island methylation parallel the heterogeneous loss of E-cad and ER expression in these tumors.

In summary, these data indicate that the malignant progression of human ductal breast carcinomas involves a heterogeneous pattern of methylation for both the ER and E-cad 5' CpG islands that begins prior to the acquisition of invasiveness, but increases for each CpG island with advancing disease. In the case of E-cadherin, these results are particularly striking because loss of E-cad expression is generally associated with the acquisition of invasive or metastatic potential rather than the earlier stages of tumorigenesis. Finally, the increase in the coincident methylation of both CpG islands suggests that malignant progression of human breast cancer involves not only the well-documented accumulation of genetic "hits" (reference), but also an accumulation of epigenetic "hits" that contribute to the diminished expression of key tumor suppressor genes like ER and E-cad.

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Table 1: Incidence of CpG island methylation for ER and E-cadherin genes in human breast tumors.

Tumor type	% ER meth.	% E-cad meth.	Coincident	+ for ER or E-cad
All	49% (54/111)	48% (53/111)	35% (39/111)	61% (68/111)
DCIS	34% (12/35)	31% (11/35)	21% (7/35)	46% (16/35)
IDC	52% (25/48)	52% (25/48)	38% (18/48)	67% (32/48)
LA/MDC	61% (17/28)	61% (17/28)	50% (14/28)	71% (20/28)
p (trend) M-H chi-square	0.034	0.019	0.013	0.032

DCIS = Ductal Carcinoma in situ
IDC = Invasive Ductal Carcinoma
LA/MDC = Locally Advanced or Metastic Ductal Carcinoma
M-H = Mantel-Haenzael

Figure Legends

Figure 1. **MSP analysis of the E-cadherin and ER CpG islands in human breast cancers.** Methylation specific PCR was used to assess the methylation status of each CpG island. **(A) DCIS.** Representative results from 6 DCIS lesions are shown. The two genes were analyzed concurrently by performing duplex PCR reactions that contained primers for both islands. **(B) IDC and MDC.** Representative results from four primary (1°)-metastatic (met) pairs are shown. Here, MSP reactions for E-cad and ER were run and analyzed separately. Metastatic sites were as follows: #1, bone; #2, chestwall; #3, axillary lymph node; #4 supraclavicular lymph node. u=primers specific for unmethylated DNA, m=primers specific for methylated DNA. Water served as a negative control and DNA from MCF-7 and MDA-MB-231cells served as positive controls for the unmethylated and methylated reactions, respectively.

Appendix 3C

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The Biology of Breast Cancer

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Synopsis

One of the most widely used and effective treatments for breast cancer is the antiestrogen tamoxifen. The efficacy of this hormonal therapy can be explained by the dependence of many breast tumors on estrogen for growth and survival. Like estrogen, many other hormones and growth factors are known to play a role in normal breast development, and have also been proposed as factors in the etiology of breast cancer. A better understanding of the contribution of these factors to breast neoplasia may provide the impetus for developing new therapies to combat inherent or acquired resistance to antiestrogens, which is common.

Introduction

Breast cancer is the most frequently diagnosed cancer among women in the western world, with approximately 180,000 new cases identified annually in the United States alone. It is currently a leading cause of cancer mortality in women, second only to lung cancer. The ultimate goal in studying breast cancer biology is to reduce mortality by identifying women at risk for the disease, predicting the prognosis of existing disease, and predicting response to different therapies. Here we will focus primarily on the latter two.

Many of the current therapies for breast cancer include standard cytotoxic agents are used to treat a wide variety of cancer types. However, one of the most widely used and effective agents in the battle against breast cancer is the antiestrogen, tamoxifen. The efficacy of this comparatively nontoxic hormonal therapy is based on the specific biology of breast cancer. About two-thirds of breast tumors express the estrogen receptor-α (ER); many of these are dependent on estrogen for growth and survival and thus respond to treatment with antiestrogens. Unfortunately the remaining one-third of breast cancers which are ER-negative at the time of diagnosis generally do not respond to endocrine therapy. Acquired resistance to tamoxifen in ER-positive tumors is also quite common. For these patients, there is clearly a need for new and better treatment options. It is widely hoped that improving our understanding of the basic biology of breast cancer will lead to the identification of new targets for the treatment or perhaps even the prevention of breast cancer. Ideally, novel therapeutic or prophylactic agents would specifically target critical biological pathways in breast tumor cells.

During the process of breast tumorigenesis, the mammary cells undergo a variety of genotypic and phenotypic changes which allow them to bypass the normal controls of tissue homeostasis. In this review, we will focus on the hormonal regulation (endocrine, paracrine, and autocrine) of breast development and how abrogations in those pathways may contribute to breast tumorigenesis by promoting inappropriate growth and survival of breast epithelial cells. Special attention will be paid to how the pathways relate to or interact with estrogen signaling, since antiestrogens have already proven themselves to be effective in the prevention and therapy of some breast cancers. Determining whether other hormonal pathways may play a role in inherent or acquired resistance to tamoxifen could lead to novel therapies which could be used in combination with tamoxifen, or perhaps after failure of tamoxifen treatment.

Breast tumorigenesis requires a variety of genetic changes such as activation or amplification of oncogenes or loss of tumor suppressor genes. Progression of the tumor to an aggressive, metastatic cancer depends on additional changes that permit invasion, migration, angiogenesis, and evasion from the immune system. Changes that promote genetic instability may also play a critical role in breast tumor progression, especially given the recent discovery that the protein products of the familial breast cancer genes BRCA1 and 2 associate with the DNA repair machinery of the cell. Many sporadic breast cancers also show altered expression of these genes. Although all of critical importance, these topics are beyond the scope of this chapter and are well reviewed elsewhere (7, 9, 22, 26, 27, 34, 74).

NORMAL MAMMARY GROWTH AND DEVELOPMENT

In order to comprehend how breast cancer cells differ from their normal counterparts, it is important to understand first the biology of the normal mammary gland. Unlike most tissues of the body that commonly complete growth and development during embryonic or juvenile phases of life, mammary tissue exhibits maximum growth potential during specific reproductive-associated cycles in adult life. The mammary gland traverses multiple cycles of proliferation,

differentiation, and regression due to repeated reproductive cycles and gestation periods (reviewed in 11, 22, 23, 43).

At birth, the mammary gland consists of a primary duct and a few branching ducts within a fat pad. Between birth and puberty, mammary tissue undergoes hormone independent isometric growth, due primarily to an increase in stromal tissue. With the onset of ovarian activity, the mammary gland goes through a phase of allometric growth. Stem cells at the epithelial end bud tips rapidly divide, differentiate, and arrest in G_O, leading to elongation and branching of the ducts. Classical endocrine ablation/replacement experiments demonstrated that ductal growth requires estrogen and either prolactin or growth hormone. Mice which lack ER due to homozygous deletion (ER knockout mice) develop only vestigial ducts at the nipples, confirming the requirement for ER function in duct formation (51). At sexual maturity, ductal development essentially stops, although the end bud epithelial tissue continues to respond to the cyclic hormonal stimuli of the menstrual cycle with alternating rounds of proliferation and apoptosis. The greatest increase in mitotic index occurs during the luteal phase of the cycle, suggesting a role for progesterone.

During pregnancy, high levels of estrogen, progesterone, and prolactin promote growth of the ducts and formation of lobuloalveolar structures. Estrogen and progesterone act synergistically to stimulate lobuloalveolar development, due in part to positive regulation of progesterone receptor expression by estrogen. Experiments with mice carrying null mutations for either progesterone or prolactin receptor have confirmed that alveolar development is dependent on the function of these receptors (12, 82). In addition to being mitogenic, progesterone inhibits initiation of lactogenesis during this stage of development. As pregnancy progresses to term, cell proliferation slows and epithelial cells differentiate in preparation for lactation.

The decrease in hormone levels and increased intramammary pressure at the time of weaning initiates involution. During the first few days, the basement membrane of the alveoli begins to break down, and extensive cell death *via* apoptosis ensues. When involution is complete, only a highly branched ductal system with some alveoli remains.

In addition to classical endocrine hormones, a number of tissue growth factors are known to influence normal mammary cell growth and function (Figure 1). For example, epidermal growth factor (EGF), transforming growth factors-ß (TGF-ß), insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), and related peptides have been implicated in the growth, development and differentiation of mammary cells. In fact, it has been widely postulated that some of the effects of estrogen on breast epithelium may be indirect and dependent upon its ability to promote the paracrine or autocrine effects of growth factor pathways *via* up-regulation of growth factor secretion and/or receptor expression. The specific contributions of the various growth factors will be discussed in more detail below.

STEROID HORMONES IN BREAST CANCER

As described above, the ovarian steroid hormones, estrogen and progesterone, are essential for normal mammary growth and development. Both interact with nuclear receptors (ER and PR) that act as gene transcription factors. Two ER genes have been identified. The first, ERα, was cloned from a human breast cancer cell line and is expressed in the normal mammary gland. Recently, a highly homologous gene (ERß) was cloned from prostate tissue (52). Although there is preliminary evidence to suggest that ERß may be expressed at low levels in some normal and malignant breast cells (112), the contribution of this expression to the

growth or survival of either normal and tumorigenic breast cells is completely undefined at present. Thus, for simplicity, ER α will be referred to as ER. A single PR gene has been identified, but at least two protein isoforms are expressed.

Estrogen

Estrogen is thought to play a significant role in the development, progression, treatment and outcome of breast cancer (1, 16, 33, 41, 50, 63, 86). Three consistently documented risk factors for breast cancer (age at menarche, menopause, and first pregnancy) are associated with the most dramatic physiological changes in estrogen secretion during a woman's lifetime. About two-thirds of primary breast tumors are ER-positive. Tumors which express ER tend to grow more slowly, are more highly differentiated, and are associated with longer disease free survival than tumors which lack ER. Clinical outcomes of breast cancer are related to ER expression because ER-positive tumors are more responsive to endocrine therapy with antiestrogens such as tamoxifen (24). Preliminary clinical results now indicate that tamoxifen and a related compound (raloxifene) may also be useful for the prevention as well as the treatment of breast cancer (18, 46, 118).

In vitro, estrogen stimulates cell cycle progression in ER positive breast cancer cells, but this action is temporally limited to early G_1 phase, suggesting that estrogen modulates expression or activity of G_1 regulatory proteins (106). Similarly, antiestrogens block proliferation during the same window of the cell cycle, with a concomitant decrease in expression of the G_1 cyclins D1 and E , and reduced phosphorylation of Rb-1, the major target of G_1 cyclin-associated kinases (84). Such observations suggest that overexpression of those cyclins, which is common in human breast cancer specimans (106), could potentially interfere with the efficacy of antiestrogen therapy. *In vivo* , antiestrogens inhibit tumor growth, in conjunction with a reduced S-phase fraction.

In addition to its role as a mitogen, estrogen can also function as a survival factor for ER positive breast tumor cells since regression of ER-positive xenograft tumors following estrogen ablation is associated with the induction of apoptosis (55). The antiapoptotic protein, Bcl-2, is commonly expressed in human breast cancer, and that expression is associated with estrogen receptor-positive tumors (reviewed in 92), suggesting that estrogen may promote survival by regulating Bcl-2 expression. In ER positive breast cancer cell lines grown *in vitro*, estrogen increases Bcl-2 levels without affecting expression of Bax, a pro-apoptotic protein (107, 115). Furthermore, estrogen-induced increases in Bcl-2 are significantly inhibited by antiestrogens.

Unfortunately, many ER positive cancers eventually become resistant to antiestrogen therapy. Defining the biological mechanisms that allow breast tumor cells to survive and grow in an estrogen independent manner could be very helpful in overcoming one of the major obstacles to effective breast cancer therapy. In most cases, the tumor cells continue to express ER, but are no longer responsive to either estrogen or tamoxifen. Several possible mechanisms may contribute to this progression, including ligand independent activation of ER, the expression of variant or mutant forms of ER, and altered expression of down-stream estrogen targets (reviewed in 30). However, up to one third of recurrent tumors arising from ER-positive primary tumors are ER-negative (54).

Since estrogen promotes the growth and survival of normal breast tissue as well as ER-positive breast cancer cells, it may seem paradoxical that ER negative tumors have a worse clinical outcome, but it has been hypothesized that ER-negative tumors are more aggressive because they have acquired the ability to bypass the ER pathways for growth and survival. In fact, re-expression of the estrogen receptor in ER-negative breast cancer cells *via* transfection with an ER

expression construct results in an inhibition of cell growth and tumorigenicity (36, 45, 119). Those results suggest that loss of ER expression or function may be an important step in the progression of some breast tumors. The molecular mechanism underlying the loss of ER gene expression is not well understood, but most ER-negative cell lines and breast cancers lack ER mRNA as well as protein (reviewed in 57). The absence of ER mRNA expression in those tumors is not due to detectable mutations, deletions or other gross structural alterations in the ER gene, suggesting that inhibition of ER gene transcription is a likely mechanism. Loss of gene transcription in the absence of mutations could be explained by epigenetic modifications that do not result in a change in the primary DNA sequence. One such mechanism that may block transcription of a gene is methylation of cytosine-rich areas, termed CpG islands, in the 5' regulatory region the genes. CpG dinucleotides occur relatively infrequently throughout most of the mammalian genome, and most sites are methylated. In contrast, CpG islands are generally found only in gene promoter regions and are usually unmethylated in normal adult tissues, with the exception of transcriptionally silent genes on the inactive X chromosome (10) and selected genes which are parentally imprinted to silence expression of one allele (62). The unmethylated status of CpG islands appears to be essential for transcription, since methylation can block transcription of downstream sequences (10, 13, 62). Anomalous patterns of DNA methylation are common in tumor cells, and many studies indicate that a variety of tumor suppressive genes are hypermethylated and transcriptionally inactive in cancer (56).

The ER gene has a CpG island in its promoter and first exon which is extensively methylated in ER-negative breast cancer cells, but remains unmethylated in all normal tissues examined (58, 59, 85). Using a very sensitive methylation specific PCR method, we have also detected ER gene methylation in a fraction of ER-positive tumors, suggesting that heterogeneity within tumor cell populations could potentially shed light on the etiology of ER-negative recurrent tumors arising from ER-positive tumors (58). Detection of ER gene methylation in primary human breast tumors suggests that this mechanism of gene silencing could contribute to the loss of ER expression and thus hormone resistance in breast cancer. It is not clear whether DNA methylation is the initiating or the integrative event in gene inactivation, but because the primary DNA sequence is not altered by methylation, it may be possible to reactivate gene expression by altering the methylation status. DNA methylation, unlike mutation, is reversible. Indeed, treatment of ER-negative cells with an inhibitor of DNA methylation (5-azacytidine or derivatives) resulted in demethylation within the ER CpG island and restored ER gene expression (31). The receptor protein was functionally active as demonstrated by its ability to activate transcription of estrogen-responsive genes.

Progesterone

The presence or absence of PR expression is an important indicator of the prospect for response to endocrine therapy. About 50% of all ER-positive breast tumors are also positive for PR. These double positive tumors exhibit the highest response rate to endocrine therapy (about 75%), whereas less than one-third of ER-positive/PR-negative tumors initially respond (63). This may simply reflect the fact that ER is a key transcription factor for the expression of PR (91). Thus, lack of PR expression in ER-positive tumors may be indicative of a non-functional or aberrantly functioning ER that is not likely to be effected by antiestrogens. By the same logic, it is not surprising that nearly all ER-negative tumors also lack PR, and rarely respond to tamoxifen.

Like ER, the loss of PR expression has been associated with hypermethylation in the regulatory region of the gene, but again, it is not known whether methylation precedes or follows transcriptional inactivation (59)

A specific role for PR in breast cancer, distinct from that of ER, has been somewhat difficult to define, but targeting the activity of this receptor can alter tumor growth (reviewed in 67, 99). Curiously, both antiprogestins and supra-physiological doses of progesterone have been reported to inhibit the growth of breast cancer. In vitro, progestins produce a biphasic growth response in PR-positive breast cancer cells, with an initial increase in cell cycle progression followed by growth arrest (106). As in the case of estrogen, cells are only sensitive to the action of progestins in the G_1 phase of the cell cycle and respond with changes in cyclin D1 expression. The initial growth spurt is accompanied by a transient increase in cyclin D1 expression, while the long term growth arrest is associated with decreased cyclin D1 expression, as well as inhibition of G_1-specific cyclin dependent kinase activity and a reduction in Rb-1 phosphorylation. Exposure to the antiprogestin, RU486, which can reduce tumor cell growth *in vivo* and *in vitro* , leads to Rb-1 hypophosphorylation and growth inhibition, but does not target cyclin D1. Rather, cell cycle arrest by that agent is accompanied by a decrease in cyclin D3 expression. In the case of antiprogestins, it has been proposed that the G_0/G_1 cell cycle arrest is associated with a differentiation pathway, since the tumor cells form dysplastic secretory glandular structures following treatment (68).

It is not known whether progestins can also act as survival factors for breast cancer cells, but progesterone can inhibit apoptosis in the normal, involuting mammary gland (29). In contrast, progesterone antagonists promote apoptosis and hence tumor regression in xenograft breast cancer models (69).

Recently, a new mechanism has been proposed for the mitogenic actions of progesterone on breast tissue during the luteal phase of the reproductive cycle (72, 95). Progestins induce growth hormone (GH) production in focal areas of hyperplastic mammary epithelium, with a concomitant increase in serum GH. Furthermore, antiprogestins block the progestin-dependent expression of mammary GH. Although it remains to be proven whether this local synthesis of GH is responsible for breast cell proliferation, both the systemic and local increase in GH levels have the potential to promote mammary growth, as will be discussed in a later section. Since breast tumors have also been shown to express GH, this mechanism could also be playing a role in the regulation of breast tumor growth (71, 111).

PEPTIDE HORMONES AND GROWTH FACTORS IN BREAST CANCER

The EGF Family

The EGF family includesfour transmembrane tyrosine kinase receptors (HER-1 or EGFR, HER-2, 3, and 4) and several growth factors including EGF, TGFα, amphiregulin, and cripto-1. These paracrine factors are produced during the proliferative phases of ductal and lobuloalveolar mammary development. Many apsects of mammary biology, including survival, proliferation, and differentiation, are now thought to be modulated by the EGF family through complex receptor heterodimerization patterns (reviewed in 25).

It has been proposed that TGFα and related growth factors may mediate the stromal-epithelial interactions involved in the indirect growth response of mammary epithelial cells (MECs) to estrogen. When grown *in vitro*, MECs proliferate in response to estrogen only when cultured in the presence of stromal cells which secrete a variety of growth factors. TGFα is a known autocrine factor for breast cancer cells grown in culture, and estrogen increases expression of TGFα and amphiregulin in ER-positive cells. Furthermore, antibodies against

TGFα or its receptor (EGFR) can block estrogen induced growth of these cells (reviewed by 21, 22).

EGF stimulates cell cycle progression of G_1-arrested MECs through induction of cyclin expression, cdk activity, and RB-1 phosphorylation with a temporal pattern similar to that induced by serum (73, 101). EGF and related peptides also act as MEC survival factors as well as mitogens. Transgenic mouse models have clearly demonstrated that TGFα can block postlactational involution (98, 102). Experiments with normal and tranformed cell lines cultured *in vitro* have confirmed that TGFα and EGF can regulate the induction of apoptosis in MECs (2, 65), by elevating Bcl-x_L levels (75). Transfection of ER-positive breast cancer cells with the receptor HER2 resulted in increased expression of Bcl-2 and Bcl-x_L, and was associated with a greater resistance to tamoxifen-induced apoptosis (53).

A variety of mouse experiments have demonstrated the ability of EGF and TGFα to promote mammary tumorigenesis (21, 22), and several members of this family (both ligands and receptors) are commonly overexpressed in human breast cancer (109). About one-third of breast tumors overexpress the receptors HER2 or EGFR. Overexpression is generally associated with ER negative tumors, high S-phase fraction, and poor prognosis (19). In cases where the receptors are overexpressed in ER-positive tumors, several small studies suggest that such overexpression may predict poor response to antiestrogens (77). Taken together, the data suggest that up-regulation of these growth factor pathways may be involved in the acquisition of a hormone independent phenotype. This hypothesis has been tested by transfecting ER-positive cell lines with HER genes under the control of constitutive promoters. Cells forced to overexpress HER2 become estrogen-independent and tamoxifen resistant (88). A similar cell line transfected with both EGFR and TGFα was also able to grow in estrogen-depleted media in vitro (70) These studies suggest that targeting the pathways of this growth factor receptor family may provide new therapeutic options for some patients for whom antiestrogen therapy is not effective. An example of such an agent is Herceptin, a humanized monoclonal antibody that targets the extracellular portion of HER2. Preliminary reports from clinical studies with Herceptin documented its efficacy against some breast cancers that express high levels of HER2, with relatively little toxicity in non-target tissues (15).

The IGF Family

The insulin -like growth factor family consists of two growth factor ligands (IGF1 and 2), two transmembrane tyrosine kinase receptors (IGFR1 and M6P/IGFR2) and at least seven IGF binding proteins (IGFBP). IGF1 and 2 both interact with IGFR1 to stimulate its tyrosine kinase activity and thereby activate multiple intracellular signaling pathways which can regulate cell growth, survival, and differentiation in many diverse tissues. In contrast, IGF2 but not IGF1 binds with M6P/IGFR2, which lacks kinase activity and has not been demonstrated to activate any intracellular pathways. IGFR2 may play a role in regulating IGF2 activity by internalizing the growth factor and transporting it to the lysosome for degradation (79)

IGF1 plays a central role in normal mammary gland development. At puberty, there is an increase in serum IGF1 levels as well as in local production of IGF1 by the mammary stromal tissue. A specific role for IGF2 in mammary development has not been defined, but in humans, circulating IGF2 levels are quite high compared to other classes of growth factors, and IGF2 is also produced locally by breast stromal cells. Thus, it is likely to modulate the activity of mammary cells that express the IGF1 receptor. Tissue specific overexpression of IGF1 in the mammary glands of transgenic mice inhibits postlactational involution (76), a result similar to

that observed in TGFα transgenics. Mice transgenic for IGF2 frequently developed mammary tumors (6), demonstrating that this signaling pathway can promote breast tumorigenesis.

IGF1 and 2 are both potent mitogens for breast cancer cells in vitro (reviewed in 105) The mitogenic action of IGF1 is synergistic with estrogen, due in part to the fact that estrogen up-regulates expression of IGFR1, while IGF1 signaling leads to phosphorylation (and thus enhanced activity) of ER. Many breast cancer cell lines express IGFR1, and interference with its expression or activity leads to inhibition of both anchorage dependent and independent growth, as well as reduced tumor growth in some xenograft models.

It is not known what portion of human breast tumors are dependent on IGFR1 signaling. However, the receptor is commonly overexpressed in the epithelial cells of breast tumors, and its ligands, IGF1 and IGF2, are often highly expressed by the stromal cells surrounding the tumor (Reviewed in 90). Furthermore, high levels of IGFR1 expression are positively correlated with estrogen receptor expression. Breast cancer cells grown in culture respond to estrogen by increasing IGFR1 expression prior to induction of proliferation, and ER-positive breast cancer cells that overexpress IGFR1 via transfection exhibit reduced dependency on estrogen and are more sensitive to low IGF concentrations. Given the above observations, it was not surprising to find that treatment with antiestrogens like tamoxifen leads to a reduction in IGFR1 signaling and down-regulation of IGFR1 mediated growth. Experiments with a xenograft model have clearly demonstrated that stromal IGF2 expression can also be dramatically reduced by antiestrogen treatment.

In spite of the potent mitogenic effects of the IGF system, elevated IGFR1 expression has been correlated with good prognosis in human breast tumors. Some retrospective studies have shown that high expression levels of IGFR1 are associated with longer disease free survival and better overall survival (Reviewed in 60). Again, such results may seem paradoxical, but these findings may simply reflect the correlation with hormone dependence and/or an association with an early disease stage. As the tumor progresses, other pathways may become more important or downstream targets of the IGF pathways may be activated by other means.

In contrast to the apparent tumor-promoting effects ascribed to the IGFR1 receptor, the IGFR2 has tumor suppressor properties, consistent with its ability to down-regulate IGF2 signaling (Reviewed in 79). In fact loss of heterozygosity (LOH) of the IGFR2 gene locus has been found in approximately 30% of both invasive and in situ breast cancers. In several cases, LOH was coupled with somatic mutations in the remaining allele, a common phenomenon in tumor suppressor inactivation. In addition to its role in IGF2 degradation, IGFR2 plays a role in the activation of a growth inhibitor, proTGFß, pointing to an additional mechanism by which this receptor may suppress tumorigenesis.

The final members of this family, the IGFBPs, are known to transport the IGFs, prolong their half lives, and influence interactions between the IGFs and their receptors (Reviewed in 80). In primary breast tumors, there is a negative correlation between ER status and IGFBP3 expression. In vitro, estrogen inhibits the production of IGFBP3 by breast cancer cells, and addition of exogenous IGFBP3 can block estrogen stimulated proliferation. Antiestrogens, on the other hand, have been reported to upregulate the expression of several IGFBPs. Taken together, these observation could again suggest that down-regulation of the IGF system might potentially play a role in the efficacy of tamoxifen. However, recent findings also point to an IGF-independent mechanism by which IGFBPs, especially IGFBP-3, can potently inhibit growth (80). This effect appears to be mediated by interaction with a poorly characterized cell-surface-

associated protein and appears to play a direct role in the growth suppressing effects of such diverse agents as TGFß and retinoic acid in breast cancer cells.

The FGF Family

The FGF family consists of four known transmembrane tyrosine kinase receptors and at least nine ligands that interact with the receptors with varying affinities. It has been hypothesized that receptor heterodimerization may contribute to the complexity of responses regulated by this family, analagous to the EGF family. Several FGFs have been localized in the developing murine mammary gland, and normal mouse MECs require FGF for growth in culture (reviewed in 22).

Several FGFs were originally identified by transformation assays, suggesting that they could potentially play an important role in tumorigenesis. One of the first indications that FGFs could promote mammary neoplasia came from studies with the mouse mammary tumor virus (MMTV). The genes for FGF3 and 4 are both frequent targets of proviral insertion and activation in viral-induced tumors, and overexpression of FGF3 alone in transgenic mice can induce tumor formation (reviewed by 47). In vitro, many human breast cancer cell lines proliferate in response to FGFs.

Expression of several FGF ligands and receptors has been observed in normal and malignant breast tissue, but expression levels vary greatly in both, so it is difficult to assess the role of these factors in breast cancer growth or to determine whether changes in expression are contributing to tumorigenesis (47). However, transfection of ER-positive breast cancer cells with either FGF1 or 4 results in estrogen independent growth and resistance to antiestrogens, both in vitro and in vivo (47, 64, 120). Increased metastatic ability and angiogenesis in vivo were reported as well, indicating that FGFs can have complex, pleiotrophic effects on breast tumor cells. A subsequent study found that paracrine effects of FGF1 on non-tumor cells could act in synergy with the mitogenic effects of estrogen, whereas autocrine FGF1 stimulation of the epithilial cancer cells was required for estrogen independent tumor growth (121).

In contrast to the apparent growth-promoting effects of FGF1, 3, and 4 on breast cancer cells, FGF2 has been reported to inhibit the growth of several human breast cancer cell lines. This inhibition appears to be due to a block in cell cycle progression via increased expression of the G_1 cdk inhibitor p21 (113), as well as an induction of apoptosis following an increase in bax expression coupled with a decrease in bcl-2 (114). It was also reported that FGF2 treatment increased the sensitivity of the cancer cells to traditional chemotherapeutic agents (113). Interestingly, two recent studies found that higher levels of FGF2 protein in primary breast tumors were associated with longer disease-free and overall survival, adding credence to the in vitro observations that FGF2 can suppress breast tumor growth (17, 118).

The TGFß Family

TGFßs (1-3) are multi-functional growth factors that interact with two interdependent serine-threonine kinase receptor subtypes. This class of peptides inhibits the growth of most epithelial cells while promoting the growth of stromal cells. In the normal mammary gland, TGFß is a critical paracrine regulator of epithelial cell growth and regression. It potently inhibits ductal elongation during gland development (96), and promotes apoptosis during postlactational involution (104).

In nontransformed mammary epithelial cells, TGFß has been reported to block expression of the S-phase-promoting cyclin A protein, but to only moderately inhibit expression of the G_1 cyclins D1, D2, E (101, 103). Activity of existing G_1 cyclin-cdk complexes was repressed by

TGFß-induced changes in the cdk inhibitor p27, thereby blocking Rb-1 phosphorylation and thus the S-phase transition. However, it should be noted that Rb-1 function does not appear to be an absolute requirement for growth arrest by TGFß in breast cancer cell lines (81).

TGFß can induce apoptosis as well as cell cycle arrest in normal and transformed MECs. Transgenic mice which overexpress TGFß show increased occurrence of apoptosis in the mammary epithelium, with a subsequent lack of secretory lobule development (44).
Several studies have reported increased TGFß expression in human breast cancer cells which have been stimulated to undergo apoptosis by a variety of factors, including cytotoxic drugs, antiestrogens, or hormone ablation (3, 4, 55, 67). However, it was not determined whether TGFß secretion was required for apoptosis induction in those systems. In mammary tumor cells which overexpress the c-myc oncogene, TGFß can promote apoptosis by blocking survival factor-dependent up-regulation of the anti-apoptotic Bcl-x_L protein (75)

Since TGFß inhibits the growth of normal mammary epithelial cells *in vivo* (20, 100) and breast cancer cells *in vitro* (110, 122), it was originally hypothesized that TGFß treatment could be used to inhibit mammary tumor growth, and mice transgenic for TGFß have demonstrated that elevated TGFß expression can suppress mammary tumor incidence (87). However, no antitumor effect was observed in xenograft tumor-bearing mice treated with exogenous TGFß (123). Furthermore, it has been demonstrated that TGFß expression is actually higher in human breast tumors than in normal mammary tissue and protein levels are positively correlated with disease progression (39).

It may be advantageous for the tumor cells to produce large quantities of TGFß because of its potential role in promoting angiogenesis (28) and invasion (97) or in suppressing the immune system (5). The breast tumor cells must therefore develop the ability to grow in the presence of relatively high concentrations of TGFß. A potential mechanism for resistance is mutation of the receptor or a change in receptor subtype (reviewed in 93). However a change in activity or expression of one of the downstream targets of the TGFß intracellular signal pathway might also be effective in blocking growth inhibition.

Determining the exact mechanism by which cancer cells become resistant to TGFß could identify novel therapeutic targets. This possibility is particularly interesting in light of the hypothesis that the therapeutic and preventative effects of antiestrogens may be mediated at least in part by up-regulation of TGFß (reviewed in 93). One study has shown that acquisition of estrogen independence *in vitro* is accompanied by resistance to TGFß (42). If that is in fact true, then targeting TGFß resistance could potentially restore sensitivity to antiestrogen therapy in some patients who have become resistant to tamoxifen.

Growth hormone and prolactin

Growth hormone (GH) and prolactin (PRL) are closely related neuroendocrine hormones. Their transmembrane receptors (GHR and PRLR) also exhibit extensive homology and belong to a class of the cytokine receptor superfamily (reviewed in 38). In fact, human GH can bind to and activate both GHR and PRLR. Several different forms of the receptors have been reported, including soluble forms consisting of only the extracellular portion which act as binding proteins. The functional significance of the various isoforms remains obscure, but a role in tissue-specific regulation of PRL function has been suggested (8)

Pituitary-derived GH plays an important role in regulating the serum-levels of IGF1 by inducing hepatic IGF1 production, and the surge of circulating IGF1 at puberty contributes to the initiation of ductal formation in the mammary gland (reviewed in 48). Animal models suggest that this GH/IGF1 axis may play a role in breast tumorigenesis as well as normal breast

development. Aging primates treated with exogenous GH exhibit a dramatic increase in mammary epithelial proliferation index and gland size that is correlated with increased serum IGF1 levels (78). GH-transgenic mice have elevated serum IGF1 levels and exhibit hyperplasia of the mammary gland, with a high frequency of breast tumor incidence (108). In contrast, mice transgenic for a GH antagonist show ductal hyperplasia. Ablation of the GH/IGF1 axis in mice with human breast cancer xenografts, or transplant of xenografts into GH-deficient mice, results in reduced tumor growth.

There is also considerable circumstantial evidence to suggest a role for the GH/IGF1 axis in human breast tumorigenesis (reviewed in 89). For example, height is positively correlated with serum IGF1 levels and is also associated with increased breast cancer risk. Two retrospective studies have found significantly higher serum IGF1 levels in women with breast cancer than in controls, especially in premenopausal women. More recently, a study using prospectively acquired blood samples has provided more direct evidence that activity of the GH/IGF1 axis is related to risk of premenopausal breast cancer (40). However, since GH production naturally decreases with age, it remains to be determined whether higher IGF1 levels during the premenopausal years may also influence the risk of breast cancer after menopause. In any case, this study suggests that drugs which target the GH/IGF1 axis may have potential use for both prevention and therapy of breast cancer.

Although the mechanism is not known, adjuvant tamoxifen therapy has been reported to suppress the pulsatile secretion of pituitary GH, and to reduce serum IGF levels. Whether these changes are necessary for tamoxifen's efficacy is not known, but such observations raise the question as to whether prevention or therapy with tamoxifen will be more effective in women with higher pretreatment IGF levels, or those that show the greatest decrease in serum IGF following tamoxifen administration (89).

It is also interesting to note that, although GH is best known for its role in the systemic GH/IGF1 axis, it also has the potential to act directly on breast tissues because local expression of both GH and GHR has been shown in the majority of normal and malignant breast tissue, as well as a variety of benign breast lesions (66, 71, 72, 111). Although GHR did not appear to be grossly overexpressed in malignant tissues, this potential autocrine/paracrine loop could be important for the growth or survival of breast tumor cells, perhaps by upregulating local IGF1 production (49), or by interaction of locally produced GH with the PRLR in the breast.

The endocrine effects of PRL on human breast tissue include the regulation of growth and differentiation of ducts and lobules, as well as the initiation and maintenance of lactation. PRL is also a mitogen for human breast cancer cells in culture, and anti-PRL reagents can inhibit the growth of those cells (35, 37). PRL also acts synergistically with ovarian steroids to promote the growth of human breast cancer xenografts in mice (61).

A role for PRL in rodent mammary tumorigenesis has been clearly demonstrated. PRL directly contributes to the etiology of both spontaneous and carcinogen-induced murine mammary carcinoma, and treatment with anti-PRL agents induces a significant therapeutic response (116)

However, its role in human breast tumorigenesis is poorly defined. PRL has been largely discounted as a determinant in breast cancer because there is a lack of correlation between circulating PRL levels and the incidence or clinical outcome of the disease, and treatments that suppress pituitary PRL release to have not been shown to improve outcome (32). However, it is now known that PRL is produced in many extrapituitary locations, including the breast (8). If

locally produced PRL acts as an autocrine/paracrine factor, then its role in tumorigenesis may be independent of circulating PRL levels.

Both PRL and its receptor are both widely expressed in normal breast tissues and the epithelial cells in breast tumors, confirming the potential for an autocrine/paracrine loop in these tissues (14, 66, 83, 94). Three studies using in situ hybridization and immunohistochemistry, or RT-PCR detected expression in nearly all samples analyzed. The fourth study using quantitative Northern analysis found a positive correlation between expression levels of PRLR and that of ER and PR in a large panel of both human breast cancer cell lines and primary tumors (83). In the same study, the authors reported that in human breast cancer cell lines, acute treatment with progestins or long term treatment with estrogen increased PRL receptors, while addition of exogenous PRL resulted in elevated PR expression. This receptor cross regulation may provide one explanation for the observed synergy among estrogen, progesterone and PRL in the control of normal and malignant breast tissue growth.

Summary

We have focused on the major hormones and growth factors for which a critical role in normal mammary growth has been clearly defined. Certainly other hormonal systems and growth factors could also be having an impact on breast cancer initiation and progression, but their exact contribution to normal and/or malignant breast cell growth is poorly delineated. Examples of such factors include somatostatin, mammostatin, mammary-derived growth inhibitor (MDGI), mammary derived growth factor-1 (MDGF-1), inhibins, activins, androgens, glucocorticoids, vitamin D, thyroid hormones, ecosinoids, and oxytocin.

Clearly the hormonal regulation of breast cancer cell growth and survival is multifaceted and very complex. In particular, the effects of estrogens and antiestrogens on breast cells may depend on their interaction with a wide variety of other pathways. In addition, these interactions may vary among individual breast tumors depending on other genetic changes in the tumor cells which have not been discussed here, such as oncogene activation and loss of tumor suppressors. A more detailed understanding about how cells circumvent a dependency on these pathways is sorely needed in order to identify new biological targets, and design novel therapies for breast cancers that are resistant to antiestrogen therapy. Such agents could be used alone, or in combination with antiestrogens to improve response to a second course of hormonal therapy.

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Figure 1: Some of the hormonal factors and their receptors that positively and negatively modulate normal or malignant breast epithelial cell growth and survival. Receptors for peptide hormones and growth factors are found in the plasma membrane, while steroid hormone receptors reside in the nucleus. There is considerable cross talk among the various pathways, and between stromal and epithelial cells. Although not shown, stromal cells also express some of the receptors found in the epithelial cells. GFs=growth factors

Appendix 3D

Abstract #3396 , Preceedings of the 90th Annual Meeting of the American Association for Cancer Research, Philadelphia, PA (1999)

DNA methyl-transferase (DMT) expression and the cell cycle in breast cancer.

Nass SJ, Ferguson AT, El-Ashry D, Nelson W, Davidson NE Johns Hopkins University, Baltimore, MD 21231 ,Georgetown University, Washington DC 20007

ER- breast cancer cells display extensive methylation of the ER gene CpG island and have elevated DMT expression compared to ER+ cells. ER+ cells expressed DMT primarily in the S phase, whereas ER- cells expressed DMT throughout the cell cycle. In addition, levels of p21(CIP1), which disrupts DMT binding to PCNA, were inversely correlated with DMT levels. The results suggest that increased DMT expression in ER- cells is due to more complex changes than a simple elevated S-phase fraction. To determine whether growth factor pathways play a role in the regulation of DMT expression in breast cancer, cell lines derived from ER+ MCF-7 cells were examined. These lines all grow in estrogen-free conditions as a result of selection or transfection. Among untransfected or control vector-transfected lines, DMT level was correlated with S-phase fraction similar to the original panel of ER+ cell lines. Overexpression of a constitutively active Raf kinase also led to increased DMT expression, but the change in expression could be fully explained by a corresponding increase in S phase fraction. Transfection with FGF1 or 4 led to increased DMT expression which could not be accounted for by a shift in S phase fraction. The elevated DMT protein expression in FGF transfectants was accompanied by a dramatic decrease in p21, again suggesting a reciprocal relationship between these two proteins. Although all of the MCF-7-derived cell lines examined grow independent of estrogen, all but one (Raf14c) express ER protein. Furthermore, the ER CpG island remained unmethylated all of these cell lines, including Raf14c. Thus, acquisition of an estrogen-independent phenotype, even in conjunction with elevated DMT levels, was not sufficient to promote ER gene silencing via CpG island methylation.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
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MCMR-RMI-S (70-1y) 26 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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